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(54) Method for producing L-lysine or L-arginine by using methanol assimilating bacterium

(57) A DNA encoding a variant of a protein, having a loop region and six hydrophobic helices and involved in excretion of L-lysine to outside of a cell, wherein the DNA encodes a mutant protein not containing the loop region that is contained in a wild-type protein and facilitates excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assim-

ilating bacterium when the DNA is introduced into the bacterium, specifically *lysE24*, is introduced into a methanol assimilating bacterium such as *Methylophilus* bacteria to improve L-amino acid productivity, especially L-lysine and L-arginine productivities.

EP 1 266 966 A2

EP 1 266 966 A2

DescriptionBackground of the Invention5 Field of the Invention

[0001] The present invention relates to techniques used in the field of microbial industry. More precisely, the present invention relates to a method for producing L-lysine or L-arginine by fermentation and a microorganism used in the production method.

10 Description of the Related Art

[0002] Amino acids such as L-lysine, L-glutamic acid, L-threonine, L-leucine, L-isoleucine, L-valine and L-phenylalanine are industrially produced by fermentation using microorganisms that belong to the genus *Brevibacterium*, *Corynebacterium*, *Bacillus*, *Escherichia*, *Streptomyces*, *Pseudomonas*, *Arthrobacter*, *Serratia*, *Penicillium*, *Candida* or the like. In order to improve the productivity of these microorganisms, strains isolated from nature or artificial mutants thereof have been used. Moreover, various techniques have been disclosed for increasing the L-amino acid producing abilities by using recombinant DNA techniques to enhance L-amino acid biosynthetic enzymes.

[0003] Productivities of L-amino acids have been considerably increased by breeding of microorganisms such as those mentioned above and improvements of production methods. However, in order to respond to further increase in demands in future, development of methods for more efficiently producing L-amino acids at lower cost have still been desired.

[0004] As methods for producing L-amino acids by fermentation of methanol, which is a fermentation raw material available in a large amount at a low cost, there have hitherto known methods using microorganisms that belong to the genus *Achromobacter* or *Pseudomonas* (Japanese Patent Laid-open (Kokai) No. 45-25273), *Protaminobacter* (Japanese Patent Publication (Kokoku) No. 49-125590), *Protaminobacter* or *Methanomonas* (Japanese Patent Laid-open No. 50-25790), *Microcyclus* (Japanese Patent Laid-open No. 52-18886), *Methylobacillus* (Japanese Patent Laid-open No. 4-91793), *Bacillus* (Japanese Patent Laid-open No. 3-505284) and so forth. The inventors of the present invention have so far developed methods for producing L-amino acids using *Methylophilus* bacteria based on breeding by artificial mutagenesis and recombinant DNA techniques (Japanese Patent Application No. 11-368097).

[0005] In recent years, there have been identified proteins that have a function of specifically excreting an L-amino acid to outside a cell of microorganism and genes therefor, and in particular, Vrljic et al. identified a gene involved in excretion of L-lysine from a *Corynebacterium* bacterium to outside of a cell (Vrljic M., Sahm H., Eggeling L., Molecular Microbiology 22:815-826 (1996)). This gene was designated as *lysE*, and it was reported that L-lysine producing ability of *Corynebacterium* bacteria could be improved by enhancing this gene in *Corynebacterium* bacteria (WO97/23597). It is also known that productivities for some L-amino acids can be improved by increasing expression amounts of amino acid excreting proteins in *Escherichia coli* (Japanese Patent Laid-open No. 2000-189180). For example, it is reported that productivities of cysteine, cysteine and so forth can be improved by enhancing expression of ORF306 gene in *Escherichia coli* (EP885962).

[0006] However, there has so far been disclosed no example of demonstrating that the excretion process of an amino acid constitutes a serious obstacle for amino acid production by fermentation of methanol using a methanol assimilating bacterium. There is also no report on any amino acid excretion gene that can provide such an excretion activity in a methanol assimilating bacterium.

[0007] Furthermore, it has not been known that the *lysE* gene has a function of excreting amino acids other than L-lysine.

Summary of the Invention

[0008] An object of the present invention is to provide a method for efficiently producing L-lysine or L-arginine by using methanol, which is available in a large amount at a low cost.

[0009] The inventors of the present invention assiduously studied in order to achieve the aforementioned object. As a result, they found that, when an L-amino acid is produced by utilizing a methanol assimilating bacterium, especially a *Methylophilus* bacterium, the excretion process of the L-amino acid to outside of cells constituted one of barriers. Further, they successfully isolated a gene that could provide excretion activity of the amino acid, especially in the microorganism, as a factor for overcoming the barrier, and found that use of the gene enabled efficient amino acid production.

[0010] The inventors of the present invention introduced the already known *lysE* gene derived from a *Corynebacterium* bacterium into a methanol assimilating bacterium and investigated its effect on the amino acid production. How-

EP 1 266 966 A2

ever, a mutation or deletion was caused in the introduced *lysE* gene and thus *lysE* could not be functioned. Since a protein responsible for such excretion exerts its function when it is incorporated into a cytoplasmic membrane, there must be suitable relationships between the protein and properties of the membrane such as its lipid composition. Therefore, it is considered difficult to obtain expression of a membrane protein of heterogenous origin in a form that exerts its function, and the above results supported it.

[0011] The inventors of the present invention obtained by chance a mutant gene that could function in a methanol assimilating bacterium during study of the aforementioned genes for excretion of L-amino acids. They also found a remarkable effect of utilization of the mutant gene in amino acid production using a methanol assimilating bacterium.

[0012] The present invention was accomplished as described above and provides the following.

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(1) A DNA encoding a variant of a protein, the protein having a loop region and six hydrophobic helices and involved in excretion of L-lysine to outside of a cell, wherein the DNA encodes a mutant protein not containing the loop region that is contained in a wild-type protein and facilitates excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assimilating bacterium when the DNA is introduced into the bacterium.

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(2) The DNA according to (1), wherein the mutant protein substantially consists only of the hydrophobic helices.

(3) The DNA according to (1) or (2), wherein the mutant protein has all of the six hydrophobic helices.

(4) The DNA according to any one of (1) to (3), which encodes a peptide containing the first to third hydrophobic helices from the N-terminus and a peptide containing the fourth to sixth hydrophobic helices from the N-terminus.

(5) The DNA according to any one of (1) to (4), wherein the protein is LysE protein.

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(6) The DNA according to (5), wherein the LysE protein is LysE protein of a coryneform bacterium.

(7) The DNA according to any one of (1) to (5), wherein the methanol assimilating bacterium is a *Methylophilus* bacterium.

(8) A DNA encoding a protein selected from the following proteins:

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(A) a protein which comprises the amino acid sequence of SEQ ID NO: 10, and

(B) a protein which comprises the amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 10 including substitution, deletion, insertion or addition of one or several amino acid residues and shows an activity for facilitating excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assimilating bacterium.

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(9) A *Methylophilus* bacterium, into which the DNA according to any one of (1) to (8) is introduced in an expressible form and has an ability to produce L-lysine or L-arginine.

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(10) A method for producing L-lysine or L-arginine, comprising culturing the *Methylophilus* bacterium according to (9) in a medium to produce and accumulate L-lysine or L-arginine in culture and collecting the L-lysine or L-arginine from the culture.

(11) The method for producing L-lysine or L-arginine according to (10), wherein the medium contains methanol as a main carbon source.

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[0013] In the present invention, the expression of "facilitating excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell" means that, when a methanol assimilating bacterium containing the DNA of the present invention is cultured in a medium, it provides an increased amount of L-lysine, L-arginine or both of these L-amino acids excreted into the medium compared with the methanol assimilating bacterium not containing the DNA of the present invention. The promotion of excretion of the L-amino acids from the inside of the cell to the outside of the cell is observed as increased concentrations of the L-amino acids accumulated in the medium during the culture of the methanol assimilating bacterium containing the DNA of the present invention compared with the concentrations provided by the methanol assimilating bacterium not containing the DNA of the present invention, which increased concentrations are provided as a result of the promotion. Further, the promotion of excretion of the L-amino acids to outside of a cell may be also observed as decrease of intracellular concentrations of the L-amino acids when the DNA of the present invention is introduced into a methanol assimilating bacterium.

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[0014] According to the present invention, L-amino acid productivity, especially L-lysine and L-arginine productivity, of a methanol assimilating bacterium can be improved.

Brief Explanation of the Drawings

[0015] Fig. 1 shows constructions of a plasmid pRStac having the *tac* promoter and plasmids pRSlysE and pRSlysE24 consisting of the plasmid pRStac inserted with the *lysE* gene or *lysE24* gene.

[0016] Fig. 2 shows construction of a plasmid pRSlysEdapA having the *lysE24* gene and *dapA** gene.

EP 1 266 966 A2

Preferred Embodiments of the Invention

[0017] Hereafter, the present invention will be explained in detail.

5 <1> DNA of the present invention

[0018] The DNA of the present invention is a DNA that promotes excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell when it is introduced into a methanol assimilating bacterium, and it is a DNA that encodes a variant of a protein involved in the excretion of L-lysine to outside of a cell of the microorganism.

10 [0019] In the present invention, the methanol assimilating bacterium is a bacterium that can grow by using methanol as a main carbon source, and it is a bacterium of which excretion of L-amino acids such as L-lysine or L-arginine to the outside of the cell is enhanced by introducing the DNA of the present invention into it. Specifically, *Methylophilus* bacteria such as *Methylophilus methylotrophus* can be mentioned. Examples of *Methylophilus methylotrophus* include the AS1 strain (NCIMB10515) and so forth. The *Methylophilus methylotrophus* AS1 strain (NCIMB10515) can be obtained from the National Collections of Industrial and Marine Bacteria (Address: NCIMB Lts., Torry Research Station 135, Abbey Road, Aberdeen AB98DG, United Kingdom).

15 [0020] The DNA of the present invention can be obtained by introducing a mutation into a DNA encoding a protein having a loop region and six hydrophobic helices and involved in excretion of L-lysine to outside of a cell to modify it so that the protein should not have the loop region or the protein should substantially consist only of the hydrophobic 20 helices. The expression of "substantially consisting only of the hydrophobic helices" means that the mutant LysE is completely deficient in the loop region or deficient in most of the loop region to such an extent that the function of the mutant LysE should not be affected.

25 [0021] One of the embodiments of the DNA of the present invention is the DNA designated as *lysE24*, which will be described in the examples mentioned later. *lysE24* is a mutant gene isolated from *Brevibacterium lactofermentum* as a homologue of the *lysE* gene reported for *Corynebacterium* bacteria. Therefore, the DNA of the present invention may also be referred to as a "mutant *lysE*" for convenience.

30 [0022] The LysE protein that is encoded by the *lysE* gene has six hydrophobic helix regions. Some of these hydrophobic regions are estimated to be transmembrane domains. It is also estimated that a region between the third and fourth regions from the N-terminus is hydrophilic and has a loop structure. In the present invention, this hydrophilic region is called a loop region. The nucleotide sequence of wild-type *lysE* and the amino acid sequence of the LysE protein of *Brevibacterium lactofermentum* are shown in SEQ ID NOS: 7 and 8. In this amino acid sequence, hydrophobic helix regions correspond to the amino acid numbers 5-20, 37-58, 67-93, 146-168, 181-203 and 211-232. The loop region corresponds to the amino acid numbers 94-145.

35 [0023] The inventors of the present invention found that the *lysE* gene exerted lethal action in a methanol assimilating bacterium, but a DNA encoding a variant of the LysE protein that did not have the loop region or substantially consisted only of the hydrophobic helices promoted the excretion of L-lysine and/or L-arginine to outside of a cell of methanol assimilating bacterium. The DNA of the present invention encodes such a mutant LysE protein that does not have such the loop region that is contained in a wild-type LysE protein or a mutant LysE protein that substantially consists only of the hydrophobic helices.

40 [0024] The aforementioned mutant *lysE* is not particularly limited so long as it has one or more hydrophobic helices and promotes excretion of L-lysine, L-arginine or both of these L-amino acids when it is introduced into a methanol assimilating bacterium. Specifically, there can be mentioned a DNA encoding a mutant LysE that has all of the first to sixth hydrophobic helices from the N-terminus. More specifically, there can be mentioned a DNA encoding a peptide containing the first to third hydrophobic helices from the N-terminus and a peptide containing the fourth to sixth hydrophobic helices from the N-terminus. The aforementioned *lysE24* is an example of the mutant *lysE* that encodes a peptide containing the first to third hydrophobic helices and a peptide containing the fourth to sixth hydrophobic helices. The *lysE24* gene is introduced by a mutation with a stop codon downstream from the region encoding the third hydrophobic helix. When a region downstream from this stop codon was deleted as described in the examples mentioned later, the *Methylophilus methylotrophus* AS1 strain to which such a *lysE24* gene is introduced did not accumulate L-lysine in the medium. From this, it is estimated that a peptide containing the first to third hydrophobic helices and a peptide containing the fourth to sixth hydrophobic helices are separately translated and function in *Methylophilus methylotrophus*. In any case, if the *lysE24* gene is introduced into a *Methylophilus* bacterium, the production amount of L-lysine or L-arginine will be improved.

45 [0025] As the microorganism that is used as an origin of such a DNA encoding a protein involved in excretion of L-lysine to outside of a cell, i.e., the *lysE* gene or its homologous gene, any microorganisms can be utilized so long as they have variants of the genes that can express the L-lysine excretion activity in a methanol assimilating bacterium. Specifically, there can be mentioned coryneform bacteria such as *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*, *Escherichia* bacteria such as *Escherichia coli*, *Pseudomonas* bacteria such as *Pseudomonas aeru-*

EP 1 266 966 A2

ginosa, *Mycobacterium* bacteria such as *Mycobacterium tuberculosis* and so forth.

[0026] When the amino acid excretion gene is enhanced in a *Methylophilus* bacterium, a recombinant DNA can be prepared by ligating its gene fragment to a vector functioning in the *Methylophilus* bacterium, preferably a multi-copy type vector, and introduced into the *Methylophilus* bacterium as a host to transform it. Alternatively, the gene can be incorporated into a transposon and introduced into chromosome. Further, it is also possible to ligate a promoter that induces strong transcription in a *Methylophilus* bacterium upstream from the gene.

[0027] The reference that discloses *lysE* (WO97/23597) shows only the case where the *lysE* gene of coryneform bacterium was introduced into a coryneform bacterium. And it mentions only L-lysine as the excreted amino acid and discloses a structure of the protein containing six transmembrane helices as a novel protein excretion system including *LysE*. However, the inventors of the present invention confirmed that *LysE* derived from coryneform bacteria did not function at all in methanol assimilating bacteria. Furthermore, the factor that could be obtained is a factor of novel type that exert the excretion activity, which has a basic structure different from that of *LysE* of coryneform bacteria in which the six transmembrane helices are constituted by one polypeptide, and this factor can no way be anticipated from the disclosure of the aforementioned patent specification that discloses *lysE*.

15 <2> *Methylophilus* bacterium of the present invention

[0028] The *Methylophilus* bacterium of the present invention is a *Methylophilus* bacterium that is introduced with the DNA of the present invention in an expressible form and has an ability to produce L-lysine or L-arginine. It can be obtained by introducing the DNA of the present invention into a *Methylophilus* bacterium that has the L-lysine or L-arginine producing ability. The *Methylophilus* bacterium of the present invention can also be obtained by imparting an L-lysine or L-arginine producing ability to a *Methylophilus* bacterium introduced with the DNA of the present invention. The *Methylophilus* bacterium of the present invention may also be one that has been imparted with an L-lysine or L-arginine producing ability by introduction of the DNA of the present invention in an expressible form.

[0029] A *Methylophilus* bacterium having the L-lysine or L-arginine producing ability can be obtained by imparting an L-lysine or L-arginine producing ability to a wild strain of a *Methylophilus* bacterium. In order to impart the L-lysine or L-arginine producing ability, there can be used methods conventionally used for breeding of coryneform bacteria, *Escherichia* bacteria and so forth, for example, acquisition of auxotrophic mutant strains, analogue resistant strains or metabolic regulation mutant strains, creation of recombinant strains in which an L-lysine or L-arginine biosynthesis system enzyme is enhanced (refer to "Amino Acid Fermentation", the Japan Scientific Societies Press [Gakkai Shuppan Center], 1st Edition, published on May 30, 1986, pp.77 to 100) and so forth. In the breeding of L-lysine or L-arginine producing bacteria, properties of auxotrophy, analogue resistance, metabolic regulation mutation and so forth may be individually imparted or two or more of them may be imparted in combination. The biosynthesis system enzyme may be individually enhanced or two or more of them may be enhanced in combination. Furthermore, the impartation of the properties including auxotrophy, analogue resistance, metabolic regulation mutation and so forth may be combined with the enhancement of biosynthesis system enzyme.

[0030] For example, L-lysine producing bacteria can be bred as mutant strains exhibiting auxotrophy for L-homoserine or L-threonine and L-methionine (Japanese Patent Publication Nos. 48-28078 and 56-6499), mutant strains exhibiting auxotrophy for inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692), or mutant strains that are resistant to oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine, γ -methyllysine, α -chlorocaprolactam, DL- α -amino- ϵ -caprolactam, α -amino-lauryllactam, aspartic acid analogue, sulfa drug, quinoid or N-lauroylleucine.

[0031] Further, L-arginine producing bacteria can be bred as mutant strains resistant to a certain agent, for example, sulfa drug, 2-thiazolealanine, α -amino- β -hydroxyvaleric acid or the like; mutant strains exhibiting auxotrophy for L-histidine, L-proline, L-threonine, L-isoleucine, L-methionine or L-tryptophan in addition to resistance to 2-thiazolealanine (Japanese Patent Laid-open No. 54-44096); mutant strains resistant to ketomalic acid, fluoromalic acid or monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); mutant strains resistant to arginol (Japanese Patent Laid-open No. 62-24075); mutant strains resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995); mutant strains resistant to 5-azauracil, 6-azauracil, 2-thiouracil, 5-fluorouracil, 5-bromouracil, 5-azacytosine, 6-azacytosine and so forth; mutant strains resistant to arginine hydroxamate and 2-thiouracil; mutant strains resistant to arginine hydroxamate and 6-azauracil (refer to Japanese Patent Laid-open No. 57-150381); mutant strains resistant to a histidine analogue or tryptophan analogue (refer to Japanese Patent Laid-open No. 52-114092); mutant strains exhibiting auxotrophy for at least one of methionine, histidine, threonine, proline, isoleucine, lysine, adenine, guanine and uracil (or uracil precursor) (refer to Japanese Patent Laid-open No. 52-99289); mutant strains resistant to arginine hydroxamate (refer to Japanese Patent Publication No. 51-6754); mutant strains exhibiting succinic acid auxotrophy or resistance to a nucleic acid base analogue (Japanese Patent Laid-open No. 58-9692); mutant strains deficient in ability to metabolize arginine and exhibiting resistance to an arginine antagonist and canavanine and auxotrophy for lysine (refer to Japanese Patent Laid-open No. 52-8729);

EP 1 266 966 A2

mutant strains resistant to arginine, arginine hydroxamate, homoarginine, D-arginine and canavanine, or resistant to arginine hydroxamate and 6-azauracil (refer to Japanese Patent Laid-open No. 53-143288); mutant strains resistant to canavanine (refer to Japanese Patent Laid-open No. 53-3586) and so forth.

[0032] Hereafter, methods for imparting or enhancing L-amino acid producing ability by enhancing an L-amino acid biosynthetic enzyme gene will be exemplified below.

[0033] L-lysine producing ability can be imparted by, for example, enhancing activities of dihydrodipicolinate synthase and aspartokinase.

[0034] Activities of dihydrodipicolinate synthase and aspartokinase in a *Methylophilus* bacterium can be enhanced by transforming the *Methylophilus* bacterium host through introduction of a recombinant DNA prepared by ligating a gene fragment encoding dihydrodipicolinate synthase and a gene fragment encoding aspartokinase with a vector that functions in the *Methylophilus* bacterium, preferably a multiple copy type vector. As a result of the increase in the copy numbers of the gene encoding dihydrodipicolinate synthase and the gene encoding aspartokinase in cells of the transformant strain, activities of these enzymes are enhanced. Hereafter, dihydrodipicolinate synthase, aspartokinase and aspartokinase III are also referred with abbreviations of DDPS, AK and AKIII, respectively.

[0035] As a microorganism providing a gene that encodes DDPS and a gene that encodes AK, any microorganisms can be used so long as they can express DDPS activity and AK activity in a microorganism belonging to the genus *Methylophilus*. Such microorganisms may be wild strains or mutant strains derived therefrom. Specifically, examples of such microorganisms include *E. coli* (*Escherichia coli*) K-12 strain, *Methylophilus methylotrophus* AS1 strain (NCIMB10515) and so forth. Since nucleotide sequences have been revealed for a gene encoding DDPS (*dapA*, Richardaud, F. et al., J. Bacteriol., 297 (1986)) and a gene encoding AKIII (*lysC*, Cassan, M., Parsot, C., Cohen, G.N. and Palte, J.C., J. Biol. Chem., 261, 1052 (1986)), these genes can be obtained by PCR using primers synthesized based on the nucleotide sequences of these genes and chromosomal DNA of microorganism such as *E. coli* K-12 as a template. As specific examples, *dapA* and *lysC* derived from *E. coli* will be explained below. However, genes used for the present invention are not limited to them.

[0036] It is preferred that DDPS and AK used for the present invention should not suffer feedback inhibition by L-lysine. It is known that wild-type DDPS derived from *E. coli* suffers feedback inhibition by L-lysine, and that wild-type AKIII derived from *E. coli* suffers suppression and feedback inhibition by L-lysine. Therefore, *dapA* and *lysC* to be introduced into a *Methylophilus* bacterium preferably encode for DDPS and AKIII having a mutation that eliminates the feedback inhibition by L-lysine, respectively. Hereafter, DDPS having a mutation that eliminates the feedback inhibition by L-lysine may also be referred to as "mutant DDPS", and a DNA encoding the mutant DDPS may also be referred to as "mutant *dapA*, or *dapA***". AKIII derived from *E. coli* having a mutation that eliminates the feedback inhibition by L-lysine may also be referred to as "mutant AKIII", and a DNA encoding the mutant AKIII may also be referred to as "mutant *lysC*".

[0037] In the present invention, DDPS and AK are not necessarily required to be a mutant. It has been known that, for example, DDPS derived from *Corynebacterium* bacteria originally does not suffer feedback inhibition by L-lysine.

[0038] A nucleotide sequence of wild-type *dapA* derived from *E. coli* is exemplified in SEQ ID NO: 1, and the amino acid sequence of wild-type DDPS encoded by the nucleotide sequence is exemplified in SEQ ID NO: 2. A nucleotide sequence of wild-type *lysC* derived from *E. coli* is exemplified in SEQ ID NO: 3, and the amino acid sequence of wild-type AKIII encoded by the nucleotide sequence is exemplified in SEQ ID NO: 4.

[0039] The DNA encoding mutant DDPS that does not suffer feedback inhibition by L-lysine may be a DNA encoding DDPS having the amino acid sequence of SEQ ID NO: 2 including substitution of a tyrosine residue for the 118-histidine residue. Further, the DNA encoding mutant AKIII that does not suffer feedback inhibition by L-lysine may be a DNA encoding AKIII having the amino acid sequence of SEQ ID NO: 4 including substitution of an isoleucine residue for the 352-threonine residue.

[0040] The plasmid used for gene cloning may be any plasmid so long as it can replicate in microorganisms such as *Escherichia* bacteria, and there can be specifically mentioned pBR322, pTWV228, pMW119, pUC19 and so forth.

[0041] The vector that functions in *Methylophilus* bacteria is, for example, a plasmid that can autonomously replicate in *Methylophilus* bacteria. Specifically, there can be mentioned RSF1010, which is a broad host spectrum vector, and derivatives thereof, for example, pAYC32 (Chistoperdov, A.Y., Tsygankov, Y.D. Plasmid, 16, 161-167 (1986)), pMFY42 (Gene, 44, 53 (1990)), pRP301, pTB70 (Nature, 287, 396, (1980)) and so forth.

[0042] In order to prepare a recombinant DNA by ligating *dapA* and *lysC* to a vector that functions in a *Methylophilus* bacteria, the vector is digested with a restriction enzyme that corresponds to the terminus of a DNA fragment containing *dapA* and *lysC*. Ligation is usually performed by using ligase such as T4 DNA ligase. *dapA* and *lysC* may be individually incorporated into separate vectors or into the same vector.

[0043] As a plasmid containing a mutant *dapA* encoding a mutant DDPS and mutant *lysC* encoding a mutant AKIII, a broad host spectrum plasmid RSFDB0 is known (WO95/16042). *E. coli* JM109 strain transformed with this plasmid was designated as AJ12396, and the strain was deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, the inde-

EP 1 266 966 A2

pendent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository) on October 28, 1993 and received an accession number of FERM P-13936. Then, it was converted to an international deposition under the provisions of the Budapest Treaty on November 1, 1994, and received an accession number of FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain by a known method.

[0044] The mutant *dapA* contained in RSFD80 has a nucleotide sequence of wild-type *dapA* shown in SEQ ID NO: 1 including substitution of T for C at the nucleotide number 597. The mutant DDPS encoded thereby has the amino acid sequence of SEQ ID NO: 2 including substitution of a tyrosine residue for the 118-histidine residue. The mutant *lysC* contained in RSFD80 has a nucleotide sequence of wild-type *lysC* shown in SEQ ID NO: 3 including substitution of T for C at the nucleotide number 1638. The mutant AKIII encoded thereby has the amino acid sequence of SEQ ID NO: 4 including substitution of an isoleucine residue for the 352-threonine residue.

[0045] In order to introduce a recombinant DNA prepared as described above into a *Methylophilus* bacterium, any method can be used so long as it provides sufficient transformation efficiency. For example, electroporation can be used (Canadian Journal of Microbiology, 43, 197 (1997)).

[0046] The DDPS activity and AK activity can also be enhanced by the presence of multiple copies of *dapA* and *lysC* on chromosomal DNA of a *Methylophilus* bacterium. In order to introduce multiple copies of *dapA* and *lysC* into chromosomal DNA of a *Methylophilus* bacterium, homologous recombination is performed by using a sequence that is present on chromosomal DNA in a multiple copy number as a target. As a sequence present on chromosomal DNA in a multiple copy number, a repetitive DNA or an inverted repeat present at the end of a transposable element can be used. Alternatively, as disclosed in Japanese Patent Laid-open No. 2-109985, multiple copies of *dapA* and/or *lysC* can be introduced into chromosomal DNA by incorporating them into a transposon and transferring it. In both of the methods, as a result of increased copy numbers of *dapA* and *lysC* in transformant strains, activities of DDPS and AK should be amplified.

[0047] Besides the above gene amplification, the DDPS activity and AK activity can be amplified by replacing an expression control sequence such as promoters of *dapA* and *lysC* with stronger ones (refer to Japanese Patent Laid-open No. 1-215280). As such strong promoters, there are known, for example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, *P_R* promoter and *P_L* promoter of lambda phage, tet promoter, *amyE* promoter, *spac* promoter and so forth. Substitution of these promoters enhances expression of *dapA* and *lysC*, and thus DDPS activity and AK activity are amplified. Enhancement of expression control sequences can be combined with increase of the copy numbers of *dapA* and *lysC*.

[0048] In order to prepare a recombinant DNA by ligating a gene fragment and a vector, the vector is digested with a restriction enzyme corresponding to the terminus of the gene fragment. Ligation is usually performed by ligase such as T4 DNA ligase. As methods for digestion, ligation and others of DNA, preparation of chromosomal DNA, PCR, preparation of plasmid DNA, transformation, design of oligonucleotides used as primers and so forth, usual methods well known to those skilled in the art can be used. Such methods are described in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989) and so forth.

[0049] In addition to the enhancement of DDPS and AK, other enzymes involved in the L-lysine biosynthesis may also be enhanced. Such enzymes include diaminopimelate pathway enzymes such as dihydrodipicolinate reductase, diaminopimelate decarboxylase, diaminopimelate dehydrogenase (refer to WO96/40934 for all of the foregoing enzymes), phosphoenolpyruvate carboxylase (Japanese Patent Laid-open No. 60-87788), aspartate aminotransferase (Japanese Patent Publication No. 6-102028), diaminopimelate epimerase and aspartic acid semialdehyde dehydrogenase, amino adipate pathway enzymes such as homoaconitate hydratase and so forth.

[0050] Aspartokinase, aspartic acid semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase and diaminopimelate decarboxylase derived from *Methylophilus methylotrophus* will be explained later.

[0051] Further, the microorganisms of the present invention may have decreased activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway for L-lysine, or may be deficient in such an enzyme. Illustrative examples of the enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway for L-lysine include homoserine dehydrogenase (see WO95/23864).

[0052] The aforementioned techniques for enhancing activities of enzymes involved in the L-lysine biosynthesis can be similarly used for L-arginine.

[0053] L-Arginine producing ability can be improved by enhancing acetylornithine deacetylase activity, N-acetylglutamic acid- γ -semialdehyde dehydrogenase activity, N-acetyl glutamokinase activity and argininosuccinase activity (Japanese Patent Publication No. 5-23750).

[0054] L-Arginine producing ability can also be improved by enhancing activity of glutamate dehydrogenase (EP 1 057 893 A1), argininosuccinate synthase (EP0 999 267 A1), carbamoyl phosphate synthetase (EP1 026 247 A1) or N-acetylglutamate synthase (refer to Japanese Patent Laid-open No. 57-5693) or by disrupting the gene encoding an arginine repressor (argR).

EP 1 266 966 A2

<3> Production of L-lysine or L-arginine

[0055] L-Lysine or L-arginine can be produced by culturing a *Methylophilus* bacterium having L-lysine or L-arginine producing ability obtained as described above in a medium to produce and accumulate L-lysine or L-arginine in culture, and collecting the L-lysine or L-arginine from the culture.

[0056] The microorganism used for the present invention can be cultured by a method usually used for culture of a methanol assimilating microorganism. The medium used for the present invention may be either a natural or synthetic medium so long as it contains a carbon source, nitrogen source, inorganic ions and other trace amount organic components as required.

[0057] If methanol is used as a main carbon source, L-lysine or L-arginine can be produced at a low cost. When methanol is used as a main carbon source, it is added to a medium in an amount of 0.001-30%. As the nitrogen source, ammonium sulfate or the like is used by adding it to the medium. Other than these, there are added small amounts of the trace amount components such as potassium phosphate, sodium phosphate, magnesium sulfate, ferrous sulfate, manganese sulfate and so forth.

[0058] The culture is performed under an aerobic condition with shaking, aeration by stirring or the like at a pH of 5-9 and a temperature of 20-45°C, and it is usually terminated within 24-120 hours.

[0059] Collection of L-lysine or L-arginine from culture can be usually attained by a combination of known methods such as those using ion exchange resin, precipitation and others.

20 Best Mode for Carrying out the Invention

[0060] Hereafter, the present invention will be explained more specifically with reference to the following examples. The reagents used in the following examples were obtained from Wako Pure Chemicals or Nakarai Tesque unless otherwise indicated. The compositions of the media used in each example are shown below. pH was adjusted with NaOH or HCl for all of the media.

(LB medium)	
Tryptic peptone (Difco)	10 g/L
Yeast extract (Difco)	5 g/L
NaCl	10 g/L
pH 7.0	

[These were steam-sterilized at 120°C for 20 minutes.]

(LB agar medium)	
LB medium	
Bacto agar	15 g/L

[These were steam-sterilized at 120°C for 20 minutes.]

(SEII medium)	
K ₂ HPO ₄	1.9 g/L
NaH ₂ PO ₄	1.56 g/L
MgSO ₄ · 7H ₂ O	0.2 g/L
(NH ₄) ₂ SO ₄	5 g/L
CuSO ₄ · 5H ₂ O	5 mg/L
MnSO ₄ · 5H ₂ O	25 mg/L
ZnSO ₄ · 7H ₂ O	23 mg/L
CaCl ₂ · 2H ₂ O	0.72 mg/L
FeCl ₃ · 6H ₂ O	9.7 mg/L
CaCO ₃ (Kanto Kagaku)	30 g/L
Methanol	2% (vol/vol)
pH 7.0	

EP 1 266 966 A2

[Except for methanol, the components were steam-sterilized at 121°C for 15 minutes. After the components were sufficiently cooled, methanol was added.]

(SEII agar medium)	
5	K ₂ HPO ₄ 1.9 g/L
10	NaH ₂ PO ₄ 1.56 g/L
15	MgSO ₄ · 7H ₂ O 0.2 g/L
	(NH ₄) ₂ SO ₄ 5 g/L
	CuSO ₄ · 5H ₂ O 5 mg/L
	MnSO ₄ · 5H ₂ O 25 mg/L
	ZnSO ₄ · 7H ₂ O 23 mg/L
	CaCl ₂ · 2H ₂ O 0.72 mg/L
	FeCl ₃ · 6H ₂ O 9.7 mg/L
	Methanol 0.5% (vol/vol)
	pH 7.0
	Bacto agar (Difco) 15 g/L

20 [Except for methanol, the components were steam-sterilized at 121°C for 15 minutes. After the components were sufficiently cooled, methanol was added.]

Example 1

25 <1> Introduction of *lysE* gene derived from *Brevibacterium* bacterium into *Methylophilus* bacterium

[0061] An *lysE* gene, which was a homologous gene of the gene facilitating excretion of L-lysine known for *Corynebacterium* bacteria, was cloned from a *Brevibacterium* bacterium, and it was attempted to express it in a *Methylophilus* bacterium.

30 (1) Construction of pRSlysE

[0062] In order to introduce *lysE* into a *Methylophilus* bacterium, a known plasmid pRS (refer to International Patent Publication in Japanese (Kohyo) No. 3-501682) was used to construct a plasmid pRSlysE for expression of *lysE*. pRS is a plasmid having the vector segment of the pVIC40 plasmid (International Patent Publication WO90/04636, International Patent Publication in Japanese No. 3-501682) and obtained from pVIC40 by deleting a DNA region encoding the threonine operon contained in the plasmid. The plasmid pVIC40 is derived from a broad host spectrum vector plasmid pAYC32 (Chistoperdov, A.Y., Tsygankov, Y.D., Plasmid, 1986, 16, 161-167), which is a derivative of RSF1010.

[0063] First, a plasmid pRStac having the *tac* promoter was constructed from pRS according to the scheme shown in Fig. 1. The pRStac plasmid was constructed as follows. The pRS vector was digested with restriction enzymes *Eco*RI and *Pst*I and added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment of 8 kilobase pairs (henceforth abbreviated as "kbp") was collected by using EASY TRAP Ver. 2 (DNA collection kit, Takara Shuzo). On the other hand, the *tac* promoter region was amplified by PCR using the pKK223-3plasmid (expression vector, Pharmacia) as a template and the primers shown in SEQ ID NOS: 1 and 2 (a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds was repeated for 30 cycles). Pyrobest DNA polymerase (Takara Shuzo) was used for PCR. The DNA fragment containing the amplified *tac* promoter was purified by using PCR prep (Promega) and then digested at the restriction enzyme sites preliminarily designed in the primers, i.e., at *Eco*RI and *Eco*T22I sites.

[0064] Then, the reaction mixture was added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment of about 0.15 kbp was collected by using EASY TRAP Ver. 2.

[0064] The digestion product of the pRS vector and the *tac* promoter region fragment prepared as described above were ligated by using DNA Ligation Kit Ver. 2 (Takara Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking.

EP 1 266 966 A2

Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes to obtain pRStac. A plasmid in which the transcription directions of the streptomycin resistance gene on the pRS vector and the tac promoter were identical to each other was selected as pRStac.

5 [0065] pRStac obtained as described above was digested with *Sse8387I* (Takara Shuzo) and *SapI* (New England Biolabs), added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel to obtain a DNA fragment of about 9.0 kbp.

10 [0066] The *lysE* gene fragment was also amplified by PCR using chromosome extracted from the *Brevibacterium lactofermentum* 2256 strain (ATCC13869) as a template and the primers shown in SEQ ID NOS: 5 and 6 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 90 seconds). Pyrobest DNA polymerase (Takara Shuzo) was used for PCR. At this time, in order that expression of the *lysE* gene should become possible in a *Methylophilus* bacterium, the primers were designed so that nucleotides of 9-15 bp from the translation initiation codon of the *lysE* gene should be replaced with a sequence that had been known to function in a 15 *Methylophilus* bacterium (Wyborn, N.R., Mills, J., Williamis, S.G. and Jones, C.W., Eur. J. Biochem., 240, 314-322 (1996)). The obtained fragment was purified by using PCR prep (Promega) and then digested with *Sse8387I* and *SapI*. The reaction mixture was added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNA's were collected by ethanol precipitation and further collected from 0.8% agarose gel.

20 [0067] The digestion product of the pRStac vector and the *lysE* gene region fragment prepared as described above were ligated by using DNA Ligation Kit Ver. 2 (Takara Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. 25 Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of nucleotide sequence to obtain pRSlysE (Fig. 1). In pRSlysE, the *lysE* gene was positioned so that its transcription direction should be the same as that of the tac promoter.

30 (2) Introduction of pRSlysE into *Methylophilus* bacterium

[0068] pRSlysE obtained as described above was introduced into *Methylophilus methylotrophicus* AS1 strain (NCIMB10515) by electroporation (Canadian Journal of Microbiology, 43, 197 (1997)). In addition, pRS was also introduced into the AS1 strain as a control in the same manner as that for pRSlysE. As a result, several thousands of 35 colonies were obtained per 1 µg of DNA with pRS used as a control, whereas only several colonies were obtained with pRSlysE.

[0069] When plasmids were extracted from transformant strains estimated to be introduced with pRSlysE and their nucleotide sequences were investigated, a spontaneous mutation was introduced in a region encoding *lysE* for all the 40 investigated plasmids, and in some cases, a nonsense mutation was introduced as the mutation, by which a codon encoding an amino acid was replaced with a stop codon that terminated the translation. In other plasmids, deletion was observed in the *lysE* gene. It was considered that, in either case, the function of *lysE* carried by such plasmids should be lost. Further, when a plasmid in which a part of the region encoding *lysE* was intentionally deleted in such a manner that the function of the *lysE* gene should be eliminated (pRSlysEΔ1) was prepared and it was attempted to introduce it into *Methylophilus methylotrophicus*, it could be introduced at a frequency equivalent to that of the pRS vector 45 used as a control.

[0070] The aforementioned pRSlysEΔ1 was a plasmid in which a region from *PvuI* (recognizes CGATCG of the 203-209th positions in SEQ ID NO: 7) site to *MluI* (recognizes ACGCGT of the 485-491st positions of the same) site present in the region encoding *lysE* was deleted, and it was constructed as follows. Specifically, pRSlysE was digested with *PvuI* and *MluI* (Takara Shuzo), added with a phenol/chloroform solution and mixed with it to terminate the reaction. 50 After the reaction mixture was centrifuged, the upper layer was collected and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel to obtain a DNA fragment of about 10 kbp. This DNA fragment was blunted-ended by using DNA Blunting Kit (Takara Shuzo). The product was ligated by itself (self-ligation) by using DNA Ligation Kit Ver. 2 (Takara Shuzo).

[0071] This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes to obtain 55

EP 1 266 966 A2

the pRSlysEΔ1 plasmid.

[0072] As described above, the introduction frequency of pRSlysE carrying the full length *lysE* gene into *Methylophilus methylotrophus* was extremely low, and only plasmids having an *lysE* mutant gene introduced with a mutation that eliminated the function could be introduced. Considering these facts in combination, it was estimated that the introduction of the *lysE* gene into *Methylophilus methylotrophus* provided lethal effect. This indicates that the *lysE* gene cannot universally function as for the excretion of L-lysine in heterogenous bacteria.

[0073] The *Methylophilus methylotrophus* AS1 strain harboring pRSlysE introduced with a mutation was applied to an SELL plate containing 20 mg/L of streptomycin and cultured overnight at 37°C. Then, the cells of about 0.3 cm² of the medium surface were scraped, inoculated into SELL production medium (20 ml) containing 20 mg/L of streptomycin, and cultured at 37°C for 34 hours with shaking. After completion of the culture, the cells were removed by centrifugation and the L-lysine concentration in the culture supernatant was determined by using an amino acid analyzer (Nihon Bunko, high speed liquid chromatography). As a result, substantially no strain was obtained in which excretion of L-lysine was enhanced in spite of introduction of the mutant *lysE* gene.

<2> Acquisition of gene providing L-lysine excretion activity in *Methylophilus* bacteria

[0074] As described in the preceding section, it was suggested that the known *lysE* gene should provide a lethal effect in *Methylophilus* bacteria, and many mutant genes of which function was lost were obtained.

[0075] During analysis of pRSlysE introduced with a mutation, a mutant *lysE* gene that functioned in *Methylophilus* bacteria could be obtained.

[0076] This mutant *lysE* gene was designated as *lysE24* gene. When the nucleotide sequence of *lysE24* gene was analyzed, it was found that this mutation was not a mutation causing amino acid substitution, but a nonsense mutation introducing a stop codon around the center of the translation region of *lysE*. It has been reported that the *lysE* gene of *Corynebacterium* bacteria encodes a membrane protein having six hydrophobic helixes (Vrijc M., Sahm H., and Eggeling L., Molecular Microbiology 22:815-826 (1996)). In contrast, it was found that, since the above *lysE24* gene was introduced with a stop codon, the protein encoded by this gene had a structure different from that of the LysE protein encoded by a wild-type *lysE* gene, and it functioned in *Methylophilus* bacteria thanks to this structure.

[0077] The result of nucleotide sequence determination of *lysE24* is shown in SEQ ID NO: 9. The nucleotide sequence of wild-type *lysE* is shown in SEQ ID NO: 7 as reference. In *lysE24*, T (thymine) was inserted after G (guanine) at the 355th position of SEQ ID NO: 7. The plasmid having this *lysE24* was designated as pRSlysE24 (Fig. 1). When pRSlysE24 was introduced anew into the AS1 strain, the plasmid could be introduced at a frequency substantially equivalent to that of pRS. In Table 1, there is shown the result of L-lysine concentration measurement for culture supernatant of the plasmid-introduced strain, which measurement was performed in the same manner as in <1>, (2).

Table 1

Strain	Production amount of L-lysine (g/L)
AS1/pRS	<0.01
AS1/pRSlysE24	0.1

[0078] The *E. coli* JM109 strain transformed with pRSlysE24 was designated as AJ13830, and this strain was deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository on June 4, 2001 and received an accession number of FERM P-18869. Then, it was converted to an international deposition under the provisions of the Budapest Treaty on May 13, 2002, 2002, and received an accession number of FERM BP-8040.

[0079] When the *lysE24* gene of which region downstream from the stop codon produced by the aforementioned mutation was deleted was introduced into the AS1 strain, the strain did not accumulate L-lysine in the medium.

Example 2: Introduction of L-lysine biosynthesis system enzyme gene and *lysE24* gene into *Methylophilus methylotrophus*

[0080] It was found that, when the *lysE24* gene was introduced into *Methylophilus methylotrophus* AS1 strain, L-lysine was accumulated in the medium. It was considered that this was caused by enhancement of the excretion of L-lysine.

[0081] The inventors of the present invention had clarified that, if an L-lysine biosynthesis gene had been enhanced by a plasmid in a *Methylophilus* bacterium, a marked amount of L-lysine had been accumulated in a medium (Japanese Patent Application No. 11-368097). Therefore, there was investigated an effect of introduction of the *lysE24* gene into

EP 1 266 966 A2

Methylophilus methylotrophicus of which L-lysine biosynthesis gene was enhanced.

<1> Construction of plasmid pRSdapA having *dapA** gene

5 [0082] There was prepared a plasmid having a gene encoding dihydrodipicolinate synthase that did not suffer feed-back inhibition by L-lysine (*dapA**) as an L-lysine biosynthesis system enzyme gene.

10 [0083] pRStac prepared in Example 1 was digested with *Sse8387I* and *Xba*I and added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel to collect a DNA fragment of about 9 kbp.

15 [0084] The *dapA** gene fragment was amplified by PCR using the known plasmid RSFD80 (refer to WO90/16042) containing that gene as a template and the primers shown in SEQ ID NOS: 3 and 4 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds). Pyrobest DNA polymerase (Takara Shuzo) was used for PCR. The obtained *dapA** fragment was purified by using PCR prep (Promega) and then digested with restriction enzymes *Sse8387I* and *Xba*I. The reaction mixture was added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNA's were collected by ethanol precipitation and separated on 0.8% agarose gel to collect a DNA fragment of about 0.1 kbp.

20 [0085] The digestion product of the pRStac vector and the *dapA** gene region fragment prepared as described above were ligated by using DNA Ligation Kit Ver. 2 (Takara Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of nucleotide sequence to obtain a pRSdapA plasmid. In pRSdapA plasmid, the *dapA** gene was positioned so that its transcription direction should be the same as that of the *lac* promoter.

30 <2> introduction of pRSlysE24 or pRSdapA into *Methylophilus methylotrophicus* AS1 strain

35 [0086] pRSdapA obtained as described above was introduced into *Methylophilus methylotrophicus* AS1 strain by electroporation. As for the obtained transformant (henceforth also referred to as "AS1/pRSdapA"), the aforementioned *Methylophilus methylotrophicus* AS1 strain introduced with pRSlysE24 (henceforth also referred to as "AS1/pRSlysE24") and the *Methylophilus methylotrophicus* AS1 strain introduced with the pRS plasmid (henceforth also referred to as "AS1/pRS") as a control, the intracellular L-amino acid concentration and the L-amino acid concentration in culture supernatant were determined.

40 [0087] Each transformant strain was cultured overnight at 37°C on an SEII plate containing 20 mg/L of streptomycin. Then, the cells of about 0.3 cm² of the medium surface were scraped, inoculated into SEII production medium (20 ml) containing 20 mg/L of streptomycin, and cultured at 37°C for 24 hours with shaking. After completion of the culture, the cells were removed from a part of the culture broth by centrifugation and the L-amino acid concentration in the culture supernatant was determined by using an amino acid analyzer. The remaining culture broth was passed through silicone oil to separate the culture broth and the cells according to the method of Kinnbier et al. (Dinnbier et al., Arch. Microbiol. 150:348-357 (1988)). Amino acids in the cells were extracted with perchloric acid, and the L-amino acid concentration was measured by using an amino acid analyzer. At this time, concentration of proteins contained in the cells was simultaneously measured, and the intracellular L-lysine concentration was represented as an amount per unit weight of the intracellular proteins.

45 [0088] The results are shown in Table 2. With AS1/pRSlysE24, L-lysine accumulation substantially equivalent to that of AS1/pRSdapA was observed in the medium. On the other hand, with AS1/pRSlysE24, the intracellular L-lysine concentration was suppressed to a low level, and it was considered that L-lysine was excreted to outside of the cells due to the introduction of the *lysE24* gene.

Table 2

strain	L-lysine concentration in culture supernatant (g/L)	intracellular L-lysine concentration (g/mg protein)
AS1/pRS	<0.01	1.60
AS1/pRSlysE24	0.10	2.80

EP 1 266 966 A2

Table 2 (continued)

strain	L-lysine concentration in culture supernatant (g/L)	intracellular L-lysine concentration (g/mg protein)
AS1/pRSdapA	0.12	17.3

[0089] Further, concentrations of other L-amino acids in the culture supernatant were also investigated. As a result, it was found that L-arginine accumulated in AS1/pRSlysE24. Thus, it was found that *lysE24* had excretion activity not only for L-lysine but also for L-arginine. The results are shown in Table 3.

Table 3

Strain	L-Arginine concentration in culture supernatant (g/L)
AS1/pRS	<0.01
AS1/pRSlysE24	0.04

[0090] The *E. coli* JM109 strain transformed with the pRSdapA plasmid was designated as AJ13831, and this strain was deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository on June 4, 2001 and received an accession number of FERM P-18370. Then, it was converted to an international deposition under the provisions of the Budapest Treaty on May 13, 2002, and received an accession number of FERM BP-8041.

<3> Introduction of *lysE24* gene and *dapA** gene into *Methylophilus methylotrophus* AS1 strain

[0091] It was found that, although the excretion of L-lysine to the outside of cells constituted a rate-limiting factor in the L-lysine production by *Methylophilus methylotrophus* AS1 strain, the excretion of L-lysine to the outside of cells was enhanced by the introduction of the *lysE24* gene. Therefore, it was attempted to further improve the productivity by enhancing the L-lysine biosynthesis enzyme system in a strain introduced with the *lysE24* gene.

(1) Construction of plasmid having *lysE24* and *dapA**

[0092] In order to evaluate effect of combination of *lysE24* and *dapA**, a plasmid consisting of the pRSlysE plasmid inserted with the *dapA** gene was constructed in the scheme shown in Fig. 2. pRSlysE24 prepared in Example 1 was digested with a restriction enzyme *Sph*I and ends of the product were blunt-ended by using DNA Blunting Kit (Takara Shuzo). Further, the plasmid pRSdapA having *dapA** was digested with restriction enzymes *Eco*RI and *Sph*I, and a fragment of about 1 kbp containing the *tac* promoter and the *dapA** region was separated on 0.8% agarose gel and collected by using EASY TRAP Ver 2 (Takara Shuzo). This fragment was blunt-ended in the same manner as described above and ligated to the aforementioned digestion product of pRSlysE24 by using DNA Ligation Kit Ver 2 (Takara Shuzo).

[0093] This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were plated on LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies appeared on the agar medium were each inoculated into LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali-SDS method and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of nucleotide sequence to obtain a pRSlysEdapA plasmid. In this plasmid, the *lysE24* gene and the *dapA** gene were positioned so that their transcription directions should be identical to each other.

[0094] pRSlysEdapA obtained as described above as well as pRSlysE24, pRSdapA and pRS plasmids as controls were each introduced into *Methylophilus methylotrophus* AS1 strain (NCIMB10515) by electroporation.

(2) Production of L-lysine by *Methylophilus* bacterium harboring *lysE24* and *dapA**

[0095] Each of the AS1 strains introduced with pRSlysEdapA, pRSlysE24, pRSdapA or pRS, which were obtained as described above, was applied to an SEII plate containing 20 mg/L of streptomycin and cultured overnight at 37°C. Then, the cells of about 0.3 cm² of the medium surface were scraped, inoculated into SEII production medium (20 ml) containing 20 mg/L of streptomycin, and cultured at 37°C for 34 hours with shaking. After completion of the culture, the cells were removed by centrifugation and the L-lysine concentration in the culture supernatant was determined by using an amino acid analyzer (Nihon Bunko, high speed liquid chromatography). The results are shown in Table 4.

EP 1 266 966 A2

5 The strain introduced with pRSlysEdapA showed L-lysine accumulation in the medium about 10 times higher than that shown by the strain introduced only with pRSdapA or pRSlysE24. Thus, it can be seen that the rate-limiting effect by the excretion was canceled by the introduction of the *lysE24* gene and the effect of the enhancement of the *dapA** gene was synergistically manifested.

5

Table 4

Strain	L-Lysine production amount (g/L)
AS1/pRS	<0.01
AS1/pRSlysE2	0.10
AS1/pRSdapA	0.12
AS1/pRSlysEdapA	1.20

10 [0096] The *E. coli* JM109 strain transformed with the pRSlysEdapA plasmid was designated as AJ13832, and this strain was deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository on June 4, 2001 and received an accession number of FERM P-18371. Then, it was converted to an international deposition under the provisions of the Budapest Treaty on 15 May 13, 2002, 2002, and received an accession number of FERM BP-8042.

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EP 1 266 966 A2

SEQUENCE LISTING

5 <110> Ajinomoto Co. Inc.

10 <120> Method for Producing L-Lysine or L-Arginine
by Using Methanol Assimilating Bacterium

15 <130> EPA-53927

20 <150> 2001-177075
<151> 2001-06-12

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EP 1 266 966 A2

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EP 1 266 966 A2

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EP 1 266 966 A2

EP 1 266 966 A2

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EP 1 266 966 A2

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 100 105 110
 55 Asp Asp Thr Pro Leu Gly Val Phe Gly Gly His
 115 120

EP 1 266 966 A2

Claims

- 5 1. A DNA encoding a variant of a protein having a loop region and six hydrophobic helixes and involved in excretion of L-lysine to outside of a cell, wherein the DNA encodes a mutant protein not containing the loop region that is contained in a wild-type protein and facilitates excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assimilating bacterium when the DNA is introduced into the bacterium.
- 10 2. The DNA according to claim 1, wherein the mutant protein substantially consists only of the hydrophobic helixes.
- 15 3. The DNA according to claim 1 or 2, wherein the mutant protein has all of the six hydrophobic helixes.
- 20 4. The DNA according to any one of claims 1-3, which encodes a peptide containing the first to third hydrophobic helixes from the N-terminus and a peptide containing the fourth to sixth hydrophobic helixes from the N-terminus.
- 25 5. The DNA according to any one of claims 1-4, wherein the protein is LysE protein.
6. The DNA according to claim 5, wherein the LysE protein is LysE protein of a coryneform bacterium.
7. The DNA according to any one of claims 1-5, wherein the methanol assimilating bacterium is a *Methylophilus* bacterium.
- 30 8. A DNA encoding a protein selected from the following proteins:
 - (A) a protein which comprises the amino acid sequence of SEQ ID NO: 10, and
 - (B) a protein which comprises the amino acid sequence of SEQ ID NO: 10 including substitution, deletion, insertion or addition of one or several amino acid residues and shows an activity for facilitating excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assimilating bacterium.
- 35 9. A *Methylophilus* bacterium, into which the DNA according to any one of claims 1-8 is introduced in an expressible form and has an ability to produce L-lysine or L-arginine.
10. A method for producing L-lysine or L-arginine, comprising culturing the *Methylophilus* bacterium according to claim 9 in a medium to produce and accumulate L-lysine or L-arginine in culture and collecting the L-lysine or L-arginine from the culture.
11. The method for producing L-lysine or L-arginine according to claim 10, wherein the medium contains methanol as a main carbon source.

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EP 1 266 966 A2

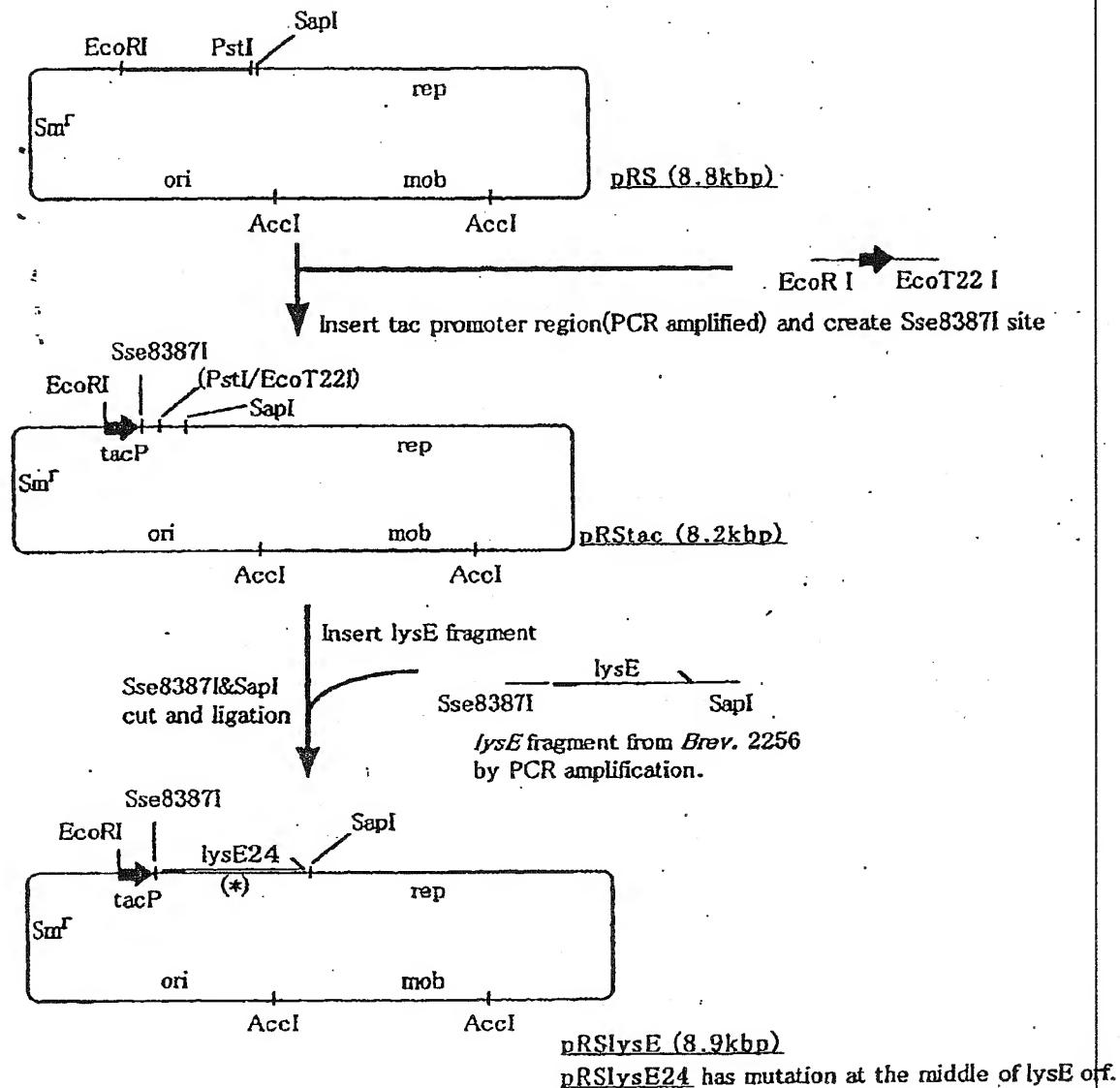


Fig. 1

EP 1 266 966 A2

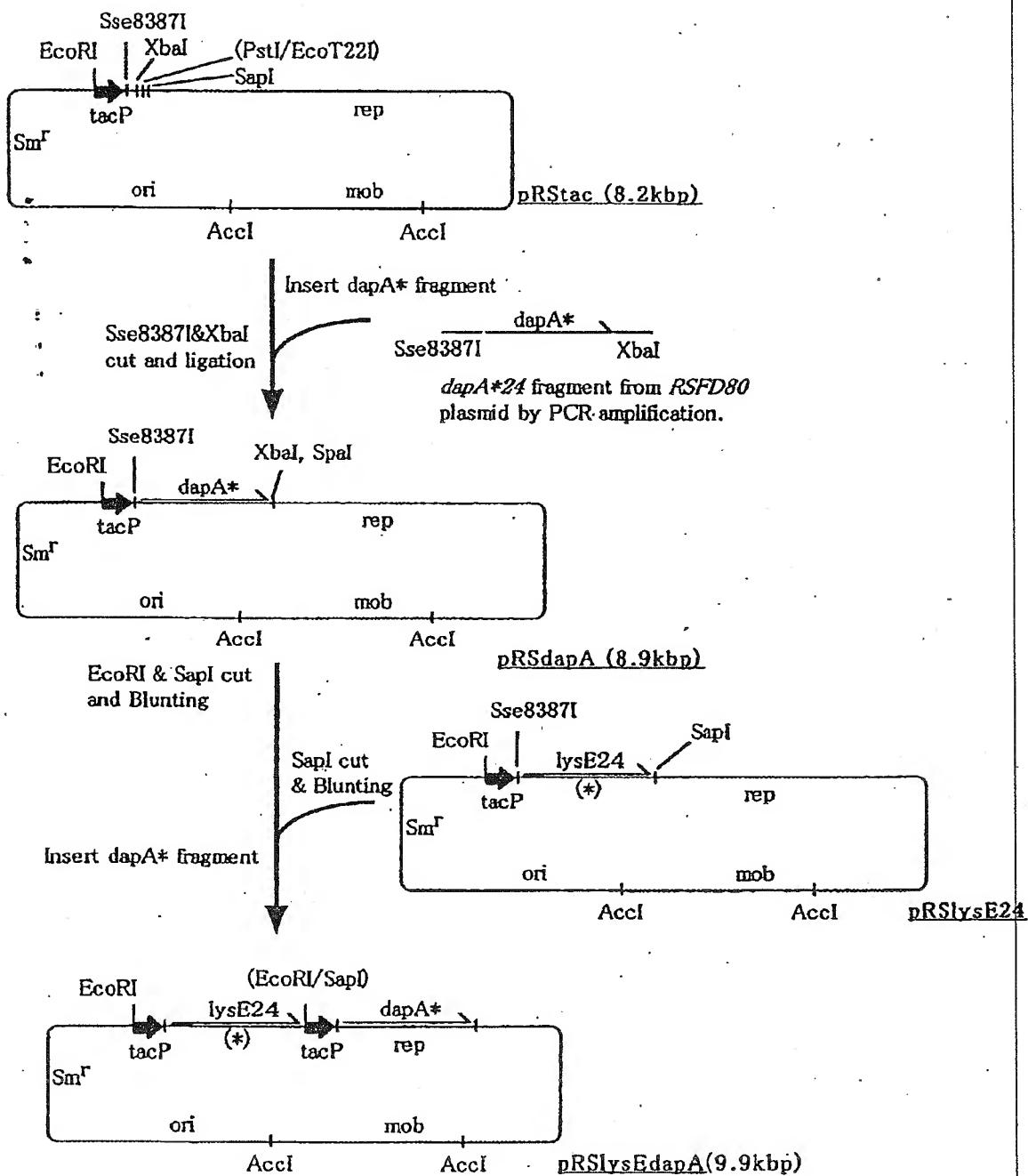


Fig. 2

(19)



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(11) EP 1 266 966 A3

(12)

EUROPEAN PATENT APPLICATION

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C12P 13/00, C12P 13/06,
C07K 14/195(43) Date of publication A2:
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(54) Method for producing L-lysine or L-arginine by using methanol assimilating bacterium

(57) A DNA encoding a variant of a protein, having a loop region and six hydrophobic helices and involved in excretion of L-lysine to outside of a cell, wherein the DNA encodes a mutant protein not containing the loop region that is contained in a wild-type protein and facilitates excretion of L-lysine, L-arginine or both of these L-amino acids to outside of a cell of a methanol assim-

ilating bacterium when the DNA is introduced into the bacterium, specifically *lysE24*, is introduced into a methanol assimilating bacterium such as *Methylophilus* bacteria to improve L-amino acid productivity, especially L-lysine and L-arginine productivities.

EP 1 266 966 A3

EP 1 266 966 A3

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EUROPEAN SEARCH REPORT

Application Number
EP 02 01 2539

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A	MOTOYAMA HIROAKI ET AL: "Characterization of the aspartate family amino acids biosynthetic enzymes in L-threonine- and L-lysine-producing mutants of <i>Methylobacillus glycogenes</i> " BIOSCIENCE BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 57, no. 3, 1993, pages 461-466, XP001160911 ISSN: 0916-8451 * the whole document *					
<table border="1"> <tr> <td colspan="2">TECHNICAL FIELDS SEARCHED (Int.Cl.7)</td> </tr> <tr> <td colspan="2">C12N C12R C12P C07K</td> </tr> </table>			TECHNICAL FIELDS SEARCHED (Int.Cl.7)		C12N C12R C12P C07K	
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Place of search	Date of completion of the search	Examiner				
THE HAGUE	21 November 2003	Hix, R				
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X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document						
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document						

EP 1 266 966 A3

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EUROPEAN SEARCH REPORT

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		-/-	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	21 November 2003	Hix, R	
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EP 1 266 966 A3



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Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
O : non-written disclosure	L : document cited for other reasons		
P : intermediate document	B : member of the same patent family, corresponding document		

EP 1 266 966 A3

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ON EUROPEAN PATENT APPLICATION NO.

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